LXXIV. EXPERIMENTAL STUDIES ON CELLULAR MULTIPLICATION.

II. THE INFLUENCE OF MUTUAL CONTIGUITY UPON REPRODUCTIVE RATE AND THE PART PLAYED THEREIN BY THE "X-SUBSTANCE" IN BACTERISED INFUSIONS WHICH STIMULATES THE MULTIPLICATION OF INFUSORIA.

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The Reproductive Rate in Cultures of two Individuals.

When two individuals from the same young parent culture of *Enchelys farcimen* are separately isolated and re-isolated, in the manner described in the preceding article, and are then introduced together into the same large drop (0·08 cc.) of hay infusion, the result is usually that not twice but from four to six times as many individuals are produced in 24 hours as in cultures of but a single individual. The following are illustrative of numerous experiments:

Normal Hay Infusion; Parent Culture 24 *hours old.*

Number of culture	Number of parent culture	Number of individuals originally introduced	Number after 24 hours	Ratio
15 B	14 B	1	12	
16 B	„	2	60	1 : 5·0
19 B	17 B	1	16	
21 B	„	2	90	1 : 5·6
36 A	35 A	1	60	
37 A	„	2	260	1 : 4·3
64 B	63 B	1	24	
65 B	„	2	102	1 : 4·2

Normal Hay Infusion; Parent Culture 48 *hours old.*

41 A	38 A	1	4	
42 A	„	2	30	1 : 7·5
41 B	38 B	1	5	
42 B	„	2	22	1 : 4·4
47 A	45 A	1	2	
48 A	„	2	8	1 : 4·0
62 A	61 A	1	8	
63 A	„	2	20	1 : 2·5
62 B	61 B	1	7	
63 B	„	2	20	1 : 2·9
188 A	184 A	1	2	
189 A	„	2	8	1 : 4·0
188 B	184 B	1	2	
189 B	„	2	11	1 : 5·5

Normal Hay Infusion; Parent Culture 72 hours old.

Number of culture	Number of parent culture	Number of individuals originally introduced	Number after 24 hours	Ratio
23 A	19 A	1	1	
24 A	„	2	6	1 : 6
23 B	19 B	1	2	
24 B	„	2	6	1 : 3
191 A	184 A	1	2	
192 A	„	2	6	1 : 3
191 B	184 B	1	1	
192 B	„	2	6	1 : 6

Pairs of individuals isolated from parent cultures older than three days either do not exhibit this phenomenon at all or only irregularly. The following is an illustrative experiment:

Normal Hay Infusion; Parent Culture in each instance Number 184.

Number of culture	Age of parent culture	Number of individuals	Individuals after 24 hours	Ratio
188 A	2 days	1	2	
189 A	2 „	2	8	1 : 4
191 A	3 „	1	2	
192 A	3 „	2	6	1 : 3
207 A	8 „	1	8	
208 A	8 „	2	16	1 : 2
188 B	2 „	1	2	
189 B	2 „	2	11	1 : 5·5
191 B	3 „	1	1	
192 B	3 „	2	6	1 : 6
207 B	8 „	1	6	
208 B	8 „	2	16	1 : 2·7

An individual isolated from an old culture and placed in the same drop of culture medium has no effect upon the reproductive rate of an individual isolated from a younger culture. The following experiment illustrates this:

Normal Hay Infusion; Parent Culture 24 hours old.

Number of culture	Number of individuals originally introduced	Number of individuals produced after 24 hours
55 B	1	14
56 B	2	48
57 B	1 from above culture plus 1 from culture 5 days old	17

The possibility that the mutually accelerative effect of two individuals is due to their conjugation may readily be dismissed. In the first place repeated observation of such cultures hour by hour up to the beginning of multiplication by fission has never revealed the occurrence of conjugation. In the second place, as Jennings has convincingly shown [Jennings, 1913], the actual effect of conjugation upon infusoria is not to accelerate but very greatly to retard their reproductive rate. Finally the only conjugants which I have ever obtained in this species have been among individuals recently washed in distilled

water, and individuals which have been so treated, as we shall see, actually fail to display the mutual accelerative effect.

At first it appeared likely that the phenomenon was simply due to the more effective seeding of the fresh hay infusion with bacteria by contamination with the parent culture fluid associated with two individuals instead of one, the available food supply being thus increased in the cultures containing two individuals. Hence many experiments were carried out upon the effects of repeated washing of the infusoria upon their ability thus mutually to influence each other's reproductive rate. The results distinctly supported the above explanation, because it was found that repeated washing in faintly alkaline water (2 cc. of $N/10$ Na_2CO_3 per 100 cc. of distilled water) completely abolished the effect. The following is one of many similar experiments:

Normal Hay Infusion; Parent Cultures Number 223 (A *and* B).

The washed individuals were suspended five successive times in 50 volumes of the faintly alkaline distilled water and recovered each time by gentle centrifugalisation which did not deform or injure them in any way.

Number of culture	Treatment of isolated individuals	Number of individuals	Individuals after 24 hours
224 A	Unwashed	1	8
225 A	Unwashed	2	24
226 A	Washed	1	4
227 A	Washed	2	5

This explanation completely broke down, however, when it was discovered that the mutual acceleration of reproductive rate by two associated individuals occurs, frequently in an even more pronounced degree, in previously bacterised infusions. The added infection carried into a bacterised hay infusion in association with the infusoria, the parent culture fluid having been diluted at least 400 times and generally many multiples of this by the new infusion, must be insignificant in comparison with the overwhelming bacterial population already present. Furthermore in bacterised infusions washing the infusoria failed to abolish or even to modify the effect. The following experiment shows this.

Normal hay infusion was bacterised by admixture with a small proportion of infusion which had been prepared 24 hours earlier and in hot weather. The parent culture was 3 days old.

Number of culture	Number of individuals	Treatment of individuals	Individuals after 24 hours
202 A	1	Unwashed	31
203 A	2	Unwashed	98
204 A	2	Washed	89

Quite convincing evidence that the accelerative effect of the second individual is not directly due to the heavier infection with associated bacteria was also afforded in another way. In the preceding article it has been pointed out that the thermal sensitivity of *Enchelys* is very great, temperatures above 35° C. being usually, and temperatures above 40° C. invariably fatal even for cultures containing many individuals. The hay bacillus is, of course, not

adversely affected by brief exposures to such temperatures. Accordingly two individuals were separately isolated and re-isolated from the same parent culture. One was killed by heating the slide by placing it for three minutes in an incubator heated to 50° C. The two small drops, one containing the living and the other the killed infusorian, were then mixed, diluted with 0·08 cc. of fresh hay infusion, and allowed to stand at room temperature as usual. Control experiments with single individuals from the same parent culture were simultaneously performed. Out of several such experiments affording similar results the following is one:

Normal Hay Infusion; Parent Culture 2 days old.

Number of culture	Individuals introduced	Number after 24 hours
242 A	1	8
243 A	2	31
244 A	1 living + 1 killed	8
242 B	1	10
243 B	2	34
244 B	1 living + 1 killed	9

Had the stimulative effect of the second individual in cultures 243 A and B been due to associated bacteria, then cultures 244 A and B should have contained at least 16 individuals each.

The question then arises why washing the infusoria cultured into fresh normal hay infusion abolishes the mutually accelerative effect. That the effect of washing is not upon the infusoria themselves, *i.e.* due to direct injury to their reproductive capacity, is shown by the fact that the mutual acceleration occurs if the washed infusoria are cultured into bacterised infusions. Evidently the loss of ability mutually to accelerate reproduction is due to the removal by washing of some associated organisms or substances which are present in abundance in bacterised infusion, although, as we have seen, the acceleration itself is not directly due to the contamination. In other words, as more extended experimentation with relatively sterile infusions now showed, *the mutual acceleration is never exhibited in the absence of a certain minimum of bacteria or their products*, although the bacteria themselves, in the absence of the second infusorian, are not able to bring about an equal acceleration of reproductive rate.

Before these facts had been ascertained, and the great importance of the bacterial population of the infusions had been established, it was not considered essential to sterilise every container used in the preparation of the hay infusions. In particular the small wide-mouthed stoppered bottle in which the infusion was placed after it was cooled and immediately before use was usually merely washed out with water between successive experiments. The occurrence of the mutually accelerative effect in "Normal Hay Infusions," so frequently displayed in the experiments cited above, was ultimately traced to this source. If the infusion is thoroughly sterile, and the infusoria are re-isolated several times, a negative result, *i.e.* absence of mutually accelerative effect, is invariably

obtained in cool weather. In hot weather, when bacteria multiply rapidly in the infusions, a negative result is less readily obtained, and in any case if the cultures be exposed to the air without a cover for some time, or any container used in preparing the medium has not been either boiled or else rinsed with ether, a positive result is usually obtained.

In some instances negative results are obtained in heavily bacterised infusions. It is not certain that this may not be due to the presence of an unfavourable type of bacterial population, but the impression has been gained, although not as yet fully confirmed, that there exists an optimum density of bacterial population for the exhibition of the effect, above which the effect dwindles and ultimately disappears.

The ability to cause mutual acceleration of reproductive rate is, as we have seen, lost with advancing age of the parent culture. It may be restored by re-culturing and sub-culturing from the young cultures. It is also gained with the growth of the individual infusoria, for a dividing individual, or two individuals which have only just completed division, when isolated into fresh hay infusion, behave like single individuals and not like pairs of individuals. The following are illustrative of a number of experiments:

Normal Hay Infusion; Parent Culture 24 *hours old.*

Number of culture	Individuals introduced	Number after 24 hours
55 A	1	14
56 A	2	56
57 A	1 (dividing)	16

Normal Hay Infusion; Parent Culture 2 *days old.*

Number of culture	Individuals introduced	Number after 24 hours
188 A	1	2
189 A	2	8
190 A	1 (dividing)	2

These experiments clearly reveal the origin of the increase of reproductive rate with population which occurs in cultures of infusoria. Doubtless the individuals arising from the division of the single isolated cell, when mature, influence each other's division-rate in just the same way as any two individuals isolated from the same parent culture. The next division multiplies the effect, and hence arise the peculiar time-relations of multiplication which have been dwelt upon in the preceding article. Since this autocatalytic increase of reproductive rate is characteristic of all congeries of living cells, we may presume that in all cases it arises out of an analogous mutual influence of contiguity. No effect of mutual contiguity can readily be imagined which is not transmitted from one cell to another, across the intervening fluid medium, through the agency of a soluble substance secreted from the cells. The existence of autogenous catalysors of cellular multiplication, therefore, must necessarily be inferred, at least in the case of infusoria, and the autocatalytic theory of growth, hitherto an hypothesis founded upon and supported by indirect evidence, has now, in a particular instance, been demonstrated to be true.

The Nature of the Substance in Bacterised Infusions which induces the Mutually Accelerative Effect.

The mutually accelerative effect of pairs of infusoria upon their reproductive rate occurs, as we have seen, only in media which contain or have contained bacteria. The actual presence of the bacteria is unnecessary, however, provided the soluble products of their metabolism are present. Filtration of the bacterised infusions through a Berkefeld filter does not abolish or even in the least diminish the accelerative effect. The following experiments show this:

Normal hay infusion was made up with every precaution to prevent infection by bacteria. A portion of this was mixed with an equal volume of an infusion which had been allowed to stand for two days at room temperature and then filtered through a Berkefeld filter. Microscopic examination showed the filtrate to be free from bacteria. The parent culture employed was three days old.

Number of culture	Culture medium	Individuals introduced	Number after 24 hours	Ratio
249 A	Normal hay infusion	1	8	
250 A	ditto	2	13	1 : 1·6
251 A	Same plus equal volume of filtered, bacterised infusion	1	14	
252 A	ditto	2	38	1 : 2·7
249 B	Normal hay infusion	1	8	
250 B	ditto	2	16	1 : 2·0
251 B	Same plus equal volume of filtered, bacterised infusion	1	10	
252 B	ditto	2	85	1 : 8·5

The effect is also obtained in pure filtered bacterised infusion, unmixed with fresh infusion, but as in this case neither bacteria nor bacterial spores are present reproduction necessarily comes to a standstill after a few divisions for lack of food. These organisms display an extremely high reproductive rate when transferred to fresh hay infusion.

The effect is not diminished by boiling the bacterised infusion, either before or after passage through a Berkefeld filter, nor is it appreciably diminished by heating the infusion for 90 minutes on a boiling water-bath. The substance which enables the effect to occur cannot be conducted by distillation from a bacterised infusion into a fresh hay infusion. In other words it resembles exactly the substance which, as shown in the preceding article, stimulates the reproductive rate of single, isolated individuals. There can be little doubt that the stimulation of reproductive rate by the "X-substance" in bacterised infusions is, in reality, not directly due to the substance itself, but to a substance produced from it, or through its agency, by the infusoria. Two infusoria produce a greater quantity than one, and the reproductive rate of each is proportionate to the concentration of this product in the medium.

Discussion of the Results.

That in media which are most favourable to rapid multiplication, contiguous cells are able to catalyse each other's reproductive rate, has been demonstrated in these experiments by direct observation of the effect. The existence of such an effect has previously been inferred from the quantitative time-relations of growth in the higher plants and animals and of multiplication in cultures of unicellular organisms, but the direct demonstration of its occurrence has also brought to light the fact that, at least in infusorial cultures, the effect is entirely dependent upon the presence of a soluble substance in the culture medium, which is produced by bacteria, and which is requisite for the manufacture of the autocatalyst by the infusoria themselves. There is, of course, no evidence as yet to show that the autocatalytic character of growth in higher animals is similarly dependent upon the presence, in the nutrient media bathing the cells, of any analogous substance, but the possibility is certainly indicated that a food-accessory may exist which is not of itself essential to growth, but which constitutes a raw material for the manufacture of growth-catalysors by the animal cells.

The "X-substance" which enables the production of growth-catalysors by infusoria is not identical with the water-soluble Vitamin B, because it resists boiling [see Robertson, 1921, p. 604]. Whether or not it is related to the fat-soluble group of accessories, or to any hitherto undiscovered accessory, remains to be ascertained.

On the other hand, given the presence of the X-substance, or raw-material, the ability of the infusoria to utilise it for the manufacture of the catalysor plainly varies with their physiological state. It diminishes during periods of starvation, or at any rate paucity of foodstuffs, and recovery is a matter of some time. The possibility may be suggested that in a densely inhabited culture, containing but few bacteria relatively to infusoria, the entire amount of catalysor produced is needed by the infusoria themselves, and remains "locked up" in their protoplasm, no excess being available for discharge into the surrounding medium. On restoration to a medium containing excess of X-substance, some time is required before a sufficient amount of catalysor can be synthesised to supply the infusoria with a sufficient content to leave a surplus for secretion into the surrounding medium. Individuals isolated from young cultures, on the other hand, start discharging excess of catalysor directly after their isolation into a medium containing the necessary supply of X-substance. The rate of production of the catalysor appears, from the autocatalytic growth-formula, to be directly proportionate to the mass of protoplasm, and this is probably the reason why a dividing infusorian, containing as it does very little greater mass of protoplasm than any other mature individual in the same culture, is able to synthesise no more of the catalysor in a given time than a single undividing individual.

SUMMARY.

1. When two individuals from the same parent culture are introduced together into the same drop of culture medium, not twice, but from four to six or eight times as many individuals are produced in 24 hours as in cultures which initially contained but a single individual.

2. The mutually accelerative effect of pairs of infusoria upon reproductive rate has been demonstrated not to be due to the more effective seeding of the culture medium by the bacteria contaminating two individuals, since it occurs equally in media which are densely inhabited by bacteria. It is also not attributable to conjugation of the associated organisms.

3. The autocatalytic theory of growth, hitherto an hypothesis founded upon and supported by indirect evidence, has thus, in a particular instance, been demonstrated to be true.

4. The accelerative effect of mutual contiguity is never exhibited excepting in the presence of soluble products of bacterial metabolism, but bacteria themselves are not essential.

5. The product which enables the effect to occur is non-volatile and thermostable. It appears to be identical with the X-substance in bacterised infusions which accelerates the multiplication of isolated single individuals.

6. The X-substance is therefore not itself an accelerator of multiplication, but is converted by animal cells into an accelerative agent, or else enables animal cells to produce an accelerative agent from other sources.

7. If the autocatalytic character of growth in the higher animals originates in a similar phenomenon, then the possibility is indicated of the existence of an accessory foodstuff which is essential, not by reason of its own direct action upon growth, but because it enables the production of autogenous catalysors of cell-multiplication by the animal cells themselves.

8. The relationship of the X-substance to the hitherto known classes of accessory foodstuffs has not yet been elucidated. It is distinguishable from the water-soluble Vitamin B, however, by its thermostability.

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REFERENCES.

Jennings (1913). *J. Exp. Zool.* **14**, 293.
Robertson (1921). *Biochem. J.* **15**, 595.